REMARKS

The claims have been amended to reduce improper multiple dependencies, without prejudice. Claims 11-20 find support throughout the specification. No new matter has been added. A marked-up copy of the amended claims is attached.

The specification has been amended to include amended pages 4-8 and the attached Sequence Listing. Amended pages 4-8 attached contain the sequence identifiers, consistent with the attached Sequence Listing. A marked up copy of pages 4-8 is also attached indicating where additions have been made. No new matter has been added.

The attached paper and computer readable copies of the Sequence Listing are the same. No new matter has been added.

An early and favorable Action on the merits is requested.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____

B. J. Sadoff
Reg. No. **36,663**

BJS:lmy
1100 North Glebe Road, 8th Floor
Arlington, VA 22201-4714
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

MARKED UP COPY OF AMENDED CLAIMS

1. (Amended) A method of preparing a medicament for treating oedema comprising admixing [Use of a peptide comprising] a chain of 7 to 17 contiguous amino acids derived from the region of human TNF-α from Ser^{100} to Glu^{116} or from the region of mouse TNF-α from Ser^{99} to Glu^{115} [for the manufacture of a medicament for treating oedema] with a pharmaceutically acceptable carrier.

2. (Amended) [Use of a peptide] A method according to claim 1, wherein said peptide comprises a chain of 11 to 16 contiguous amino acids.

3. (Amended) [Use of a peptide] A method according to claim 1, wherein said peptide comprises a chain of 13 to 15 contiguous amino acids.

4. (Amended) [Use of a peptide] A method according to claim 1, wherein said peptide comprises a chain of 14 contiguous amino acids.

5. (Amended) [Use of a peptide] A method according to claim 4, wherein said chain of 14 contiguous amino acids are chosen from the group consisting of the contiguous amino acid sequences

QRETPEGAEAKPWY and PKDTPEGAELKPWY.

6. (Amended) [Use of a peptide] A method according to [any of claims 1 to 5]

claim 1, wherein said peptide is circularized.

7. (Amended) [Use of a peptide] A method according to claim 6, wherein said peptide is circularized by replacing the NH_2- and COOH-terminal amino acids by cysteine so that a disulfide bridge is formed between the latter cysteines.

8. (Amended) A method [Use of a peptide] according to claim 7, wherein said circularized peptides are chosen from the group consisting of the circularized peptides CGQRETPEGAEAKPWYC and CGPKDTPEGAELKPWYC.

9. (Amended) A method [Use of a peptide] according to [any of claims 1 to 8] claim 1, wherein said oedema is pulmonary oedema.

10. (Amended) A pharmaceutical composition for treating oedema comprising [a peptide according to any of claims 1 to 9] a chain of 7 to 17 contiguous amino acids derived from the region of human TNF-α from Ser^{100} to Glu^{116} or from the region of mouse TNF-α from Ser^{99} to Glu^{115} and a pharmaceutically acceptable carrier.

Mark-up Sheet

indicate the means of ≥ 5 cells ± SEM (*: $P \leq 0.05$).

Figure 4: **(A)** Effect of Ltip (100 μg/ml) *versus* controls in CBA lung MVEC at pH 6 and pH 7.3. **(B)** Comparison of the effect of 30 min preincubation of MVEC with Ltip peptide, mutTip peptide, and scramblTip peptide at pH 6. Effect of amiloride (100 μM) added during the preincubation, on Ltip peptide-induced increase in membrane conductance in MVEC. Values indicate the means of ≥ 5 cells ± SEM (*: $P \leq 0.05$).

Figure 5: Effect of mTNF tip peptide (1mg/lung) on lung weight change (in g) during an isolated lung perfusion experiment lasting 150 min.

Figure 6: Effect of wild type mTNF (●,1μg/lung) or mTNF tip peptide (▲,1mg/lung) *versus* controls [O, NaCl] on lung weight change (in % versus baseline lung weight at 30 min) during isolated lung perfusion experiments after 150 min. Each symbol [O, ● or ▲] represents one lung.

DETAILED DESCRIPTION OF THE INVENTION

The invention described herein draws on previously published work and pending patent applications. By way of example, such work consists of scientific papers, patents or pending patent applications. All these publications and applications, cited previously or below are hereby incorporated by reference.

The present invention relates to the use of a peptide comprising a chain of 7 to 17, preferably a chain of 11 to 16, more preferably a chain of 13 to 15 and most preferably a chain of 14 contiguous amino acids derived from the region of human TNF-α from Ser^{100} to Glu^{116} or from the region of mouse TNF-α from Ser^{99} to Glu^{115} for the manufacture of a medicament for treating oedema. More specifically the present invention relates to the use of a peptide as described above wherein said chain of 14 contiguous amino acids are chosen from the group consisting of the contiguous amino acid sequences QRETPEGAEAKPWY (SEQ ID NO 1) and PKDTPEGAELKPWY (SEQ ID NO 2) as described by Lucas *et al.* (1994). The latter sequences are given in the well-known one-letter code for amino acids (the three-letter code is sometimes used further).

The term "peptide" refers to a polymer of amino acids (aa) derived from the trypanolytic TNF domain having lectin-like affinity as described by Lucas *et al.* (1994). Moreover, the latter term relates to a polymer of 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 or 17 contiguous amino acids derived from

the region of human TNF-α from Ser^{100} to Glu^{116} or from the region of mouse TNF-α from Ser^{99} to Glu^{115}. The latter TNF regions also refer to the regions shown in Fig. 5, p. 172 of Pennica and Goeddel in Webb and Goeddel, eds. (1987). However, it should be clear that the region of human TNF-α from Ser^{100} to Glu^{116} is identical to human TNF-α from Ser^{99} to Glu^{116} in Fig. 5, p. 172 of Pennica and Goeddel in Webb and Goeddel, eds. (1987) and that the region of mouse TNF-α from Ser^{99} to Glu^{115} is identical to mouse TNF-α from Ser^{98} to Glu^{115} in Fig. 5, p. 172 of Pennica and Goeddel in Webb and Goeddel, eds. (1987). The term "peptide" more specifically relates to a peptide comprising the hexamer TPEGAE (SEQ ID NO 3) of the latter TNF regions or any peptide comprising the corresponding amino acids T, E and E of the latter hexamer which were shown to be three critical amino acids by Lucas *et al.* (1994). It should be clear that the present invention relates to any peptide derived from the latter TNF regions and does not exclude post-translational modifications of the peptides such as glycosylation, acetylation, phosphorylation, modifications with fatty acids and the like. Included within the present invention are, for example, peptides containing one or more analogues of an aa (including unnatural aa's), peptides with substituted linkages, mutated versions or natural sequence variations of the peptides, peptides containing disulfide bounds between cysteine residues, as well as other modifications known in the art. The peptides of the present invention are also defined functionally, that is, the present invention relates to any peptide which can be used to treat oedema or which can be used for the manufacture of a medicament for treating oedema. In essence, the present invention relates to any molecule, obtained by any method known in the art, with the same or very similar characteristics as the trypanolytic peptides defined by Lucas *et al.* (1994).

The peptides of the present invention can be prepared by any method known in the art such as classical chemical synthesis, as described by Houbenweyl (1974) and Atherton & Shepard (1989), or by means of recombinant DNA techniques as described by Maniatis et al. (1982) and, more specifically, by Lucas et al. (1994).

The term oedema (or edema) relates to any abnormal excess accumulation of (serous) fluid in connective tissue or in a serous cavity. In particular, the latter term relates to pulmonary oedema (see also *Examples* section).

Furthermore, the present invention concerns the use of a peptide as described above wherein said peptide is circularized. More specifically, the present invention relates to the use of a peptide as described above, wherein said peptide is circularized by replacing the NH_2- and COOH-terminal amino acids by cysteine so that a disulfide bridge is formed between the latter cysteines. In this regard, the present invention concerns the use of a peptide as described above wherein said

circularized peptides are chosen from the group consisting of the circularized peptides CGQRETPEGAEAKPWYC (SEQ ID NO 4) and CGPKDTPEGAELKPWYC (SEQ ID NO 5) as described by Lucas *et al.* (1994).

The present invention finally relates to a pharmaceutical composition for treating oedema comprising a peptide as described above. The terms "a pharmaceutical composition for treating oedema" relates to any composition comprising a peptide as defined above which prevents, ameliorates or cures oedema, in particular pulmonary oedema. More specifically, the terms "a pharmaceutical composition for treating oedema" or "a drug or medicament for treating oedema" (both terms can be used interchangeably) relate to a composition comprising a peptide as described above and a pharmaceutically acceptable carrier or excipient (both terms can be used interchangeably) to treat oedema. Suitable carriers or excipients known to the skilled man are saline, Ringer's solution, dextrose solution, Hank's solution, fixed oils, ethyl oleate, 5% dextrose in saline, substances that enhance isotonicity and chemical stability, buffers and preservatives. Other suitable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids and amino acid copolymers. The "medicament" may be administered by any suitable method within the knowledge of the skilled man. The preferred route of administration is parenterally. In parenteral administration, the medicament of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with the pharmaceutically acceptable excipients as defined above. However, the dosage and mode of administration will depend on the individual. Generally, the medicament is administered so that the peptide of the present invention is given at a dose between 1 µg/kg and 10 mg/kg, more preferably between 10 µg/kg and 5 mg/kg, most preferably between 0.1 and 2 mg/kg. Preferably, it is given as a bolus dose. Continuous infusion may also be used. If so, the medicament may be infused at a dose between 5 and 20 µg/kg/minute, more preferably between 7 and 15 µg/kg/minute.

The present invention will now be illustrated by reference to the following examples which set forth particularly advantageous embodiments. However, it should be noted that these embodiments are illustrative and can not be construed as to restrict the invention in any way.

EXAMPLES MARKED-UP SHEET

Example 1:

MATERIAL AND METHODS

Animals, cells and reagents. Male CBA/J or C57BL/6 mice, as well as male TNFR ½ $^{0/0}$ C57BL/6 mice deficient in TNF receptors (Bruce et al., 1996) provided by H. Bluethmann, F. Hoffmann-La Roche, Basel, Switzerland, were used at the age of 8-10 weeks. Their care was in accordance with institutional guidelines. Lung microvascular endothelial cells were isolated from CBA/J mice and characterized as described (Jackson et al., 1990) using magnetic beads (Dynabeads M-450, Dynal, Oslo, Norway), covalently bound to a purified rat-anti-mouse PECAM-1 monoclonal antibody (donated by B. Imhof, University of Geneva). Microvascular lung endothelial cells were resuspended in DMEM containing 2 mM L-glutamine, 100 U/ml penicillin, 10 mg/ml streptomycin, 20% FCS, 40 U/ml heparin and 100 mg/ml endothelial cell growth supplement (Brunschwig Chemie, Basel, Switzerland). For patch clamp experiments, cells were plated onto 35x10 mm easy grip Petri dishes (Beckton Dickinson, Plymouth, UK), pre-coated with 0.2% gelatin (Sigma, Buchs, Switzerland). Resident peritoneal macrophages, isolated in ice cold RPMI containing antibiotics and 10 U/ml Heparin, were left to adhere onto 35x10 mm easy grip Petri dishes for 4 h, after which the non-adherent cells were removed. Cells were grown in RPMI 1640 containing 2 mM L-glutamine, 100 U/ml penicillin, 10 µg/ml streptomycin and 10 % fetal bovine serum (all from Gibco). For patch clamp, the macrophages were used 24 h after isolation.

TNF and peptides. *E.coli*-derived recombinant murine TNF (further referred as TNF in the text) and an *E.coli*-derived recombinant (T104A-E106A-E109A) triple TNF mutant (mutTNF) were synthesized as described elsewhere (Lucas et al., 1997). TNF-derived peptides were synthesized with the use of Fmoc-a-amino group protection (Fields et al. 1990), and purified with a C18 reversed-phase high-performance liquid chromatography column.

The following TNF-derived peptides were synthesized:

Long tip peptide 99-115 (LTip) GG-CGPKDTPEGAELKPWYC (SEQ ID NO 6)

MARKED-UP SHEET

Mutated tip peptide 99-115 (mutTip)	GG-CGPKDAPAGAALKPWYC (SEQ ID NO 7)
Scrambled tip peptide (scamblTip)	GG-CGTKPWELGPDEKPAYC (SEQ ID NO 8)
Short tip peptide (STip)	CTPEGAEC (SEQ ID NO 9)

To theoretically retain the original TNF conformation as much as possible, Ltip, MutTip and ScamblTip peptides were circularized. Ser^{99} of the TNF sequence was replaced by Cys, and Cys^{100} by Gly so that the disulfide bridge could be formed between Cys^{99} and Cys^{115} in the peptides. The STip peptide could not be circularized. The peptides were NH_2-biotinylated.

Electrophysiology. Cells were pretreated for 30 min with TNF, mutTNF and tip peptides at 37° C in a buffer consisting of 145 mM NaCl, 3 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM D-glucose, and 10 mM Hepes, and pH-adjusted with NaOH to required value. Cells were then washed with the same buffer pH-adjusted at 7.3, and experiments were performed using the tight-seal, whole-cell recording technique. Currents were recorded with an Axopatch-200A amplifier (Axon Instrument Inc, Foster City, CA, USA), low pass-filtered at 1 kHz. Digitalization and off-line analysis was performed using the WCP program (J. Dempster, Strathclyde Electrophysiology Software, Glasgow, UK). Patch pipettes were pulled from borosilicate glass and fire polished to have an open resistance of 3-5 MW with an internal solution containing 130 mM CsCl, 2 mM MgCl2, 10 mM EGTA, 20 mM TEA-Cl, 10 mM D-glucose, 10 mM Hepes, pH-adjusted to 7.3 with CsOH. Series resistances were kept under 10 MW. Capacitance and series resistance compensation were applied and set to 70%. All experiments were done at room temperature. Results are given as mean ± SEM, unless otherwise indicated. Analysis of variance was performed on currents and membrane conductance values, with post-hoc Dunn-Bonferroni test for significance of differences observed between two groups. A P value of 0.05 was considered significant.

Tryptophan fluorescence. Fluorescence measurements were made with a PTI spectrofluorimeter. The excitation wavelength was 295 nm and slit widths were 5 nm and 2.5 nm for excitation and emission respectively. For each recorded spectrum, the Raman scatter contribution was removed by subtraction of a buffer blank. All buffers contained 150 mM NaCl, and 20 mM of N-[2-morpholino] ethane-sulfonic acid (MES) buffer at the desired pH. The